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Short Article

Phospho-Regulation of the Cdc14/Clp1 Phosphatase Delays Late Mitotic Events in *S. pombe*

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Summary

In eukaryotes, exit from mitosis occurs through the inactivation of the Cdk1-cyclin B kinase complex and the reversal of its phosphorylation events. These late mitotic events are tightly regulated to occur only after the onset of anaphase and prior to cytokinesis. Central to this regulation is the conserved Cdc14 family of protein phosphatases, whose activity reverses Cdk-dependent phosphorylation events. *S. cerevisiae* Cdc14 activity is restrained from dephosphorylating Cdk substrates and inactivating Cdk1 through its nucleolar sequestration prior to anaphase. Here, we describe a unique mode of Cdc14 regulation that operates prior to anaphase in fission yeast. Cdk1 phosphorylates and inhibits the catalytic activity of the Cdc14 family member, Clp1/Flp1. As Cdk1 activity declines during anaphase progression, Clp1/Flp1 autocatalytically reverses these phosphorylation events to stimulate its own activity. These findings point to a simple regulatory circuit that couples Cdk1 activation with its inactivation mediated through phosphorylation-dependent regulation of Clp1/Flp1 phosphatase activity.

Introduction

Cdc14 protein phosphatases antagonize Cdk-dependent phosphorylation events and play key roles in coupling nuclear and cytoplasmic divisions. *S. cerevisiae* Cdc14 reverses inhibitory phosphorylation events present on Cdk1 inhibitors in late mitosis and, therefore, is essential for the complete abrogation of Cdk1-cyclin B activity at mitotic exit. Its activity is largely controlled at the level of subcellular localization, where a number of signaling pathways maintain Cdc14 in a nucleolar bound (inactive) or nucleolar released (active) state. Within the nucleolus, Net1/Cfi1 acts as a competitive inhibitor of Cdc14 activity by binding tightly to the catalytic core of the phosphatase (Shou et al., 1999; Visintin et al., 1999; Traverso et al., 2001). Disruption of this inhibitory complex through *CDC14* overexpression or loss of *net1/cfi1* function leads to Cdk1 inactivation irrespective of cell cycle stage (Visintin et al., 1998, 1999). The Cdc14-Net1/Cfi1 interaction is disrupted at anaphase onset through Cdk1-dependent phosphorylation of Net1/Cfi1 mediated by the FEAR (*f*ourteen *e*arly *a*naphase *r*elease) network, leading to release of Cdc14 from the nucleolus (Stegmeier et al., 2002; Pereira et al., 2002; Yoshida et al., 2002; Azzam et al., 2004). Nucleolar release of Cdc14 is then sustained during anaphase progression by the mitotic exit network (MEN), a GTPase signaling cascade that becomes active upon spindle elongation into the daughter bud (Jaspersen et al., 1998; Bardin et al., 2000; Pereira et al., 2000). Because separase is an essential component of the FEAR network (Stegmeier et al., 2002) and because MEN activation is inhibited by elevated Cdk1 activity (Jaspersen and Morgan, 2000; Hwa Lim et al., 2003), Cdc14 nucleolar release and activation cannot occur until the onset of anaphase, thus ensuring proper temporal activation of Cdc14.

While *Sc*Cdc14 is retained in the nucleolus until anaphase, other family members are not regulated in the same manner. Human Cdc14 isoforms A and B and *Xenopus* Cdc14A isoforms become dispersed throughout the cell upon entry into mitosis (Mailand et al., 2002; Kaiser et al., 2002, 2004). Similarly, the Cdc14 phosphatase in *S. pombe*, Clp1/Flp1 (hereafter referred to as Clp1), is released from the nucleolus early in mitosis, during which time it concentrates at the kinetochores and the contractile ring and becomes dispersed throughout the nucleus and cytoplasm (Cueille et al., 2001; Trautmann et al., 2001, 2004). Despite its dispersal from the nucleolus prior to anaphase, Clp1 substrates such as the Cdc25 phosphatase are not dephosphorylated until mitotic exit (Wolfe and Gould, 2004; Esteban et al., 2004). These observations suggested that an additional mechanism(s) might restrain Clp1 and perhaps metazoan Cdc14 activities prior to anaphase.

Here, we describe mitotic-specific phosphorylation of Clp1 by the mitotic regulator, Cdk1, and show that the result of this phosphorylation is inhibition of Clp1 catalytic activity. As Cdk1 activity falls during anaphase progression, Clp1 autocatalytically reverses these phosphorylation events to restore full activity, thus allowing subsequent dephosphorylation of other Cdk1 substrate(s).

Results

Cdk1 Phosphorylates Clp1 during Early Mitosis

As previously reported (Cueille et al., 2001), Clp1 becomes hyperphosphorylated as cells progress through mitosis (Figure 1C) and accumulates in the hyperphosphorylated state during a prometaphase arrest imposed by the β-tubulin mutant *nda3-KM311* (Figure 1A). To identify mitotic phosphorylation sites on Clp1, we purified Clp1 using a TAP tag from *nda3-KM311* arrested cells. The purification eluate was digested with proteases, and the peptides were sequenced by 2D-LC tandem mass spectrometry (MacCoss et al., 2002). SEQUEST analysis identified six Ser and Thr phosphorylation sites corresponding to minimum Cdk consensus phosphorylation sites (Ser/Thr-Pro), the majority of which were

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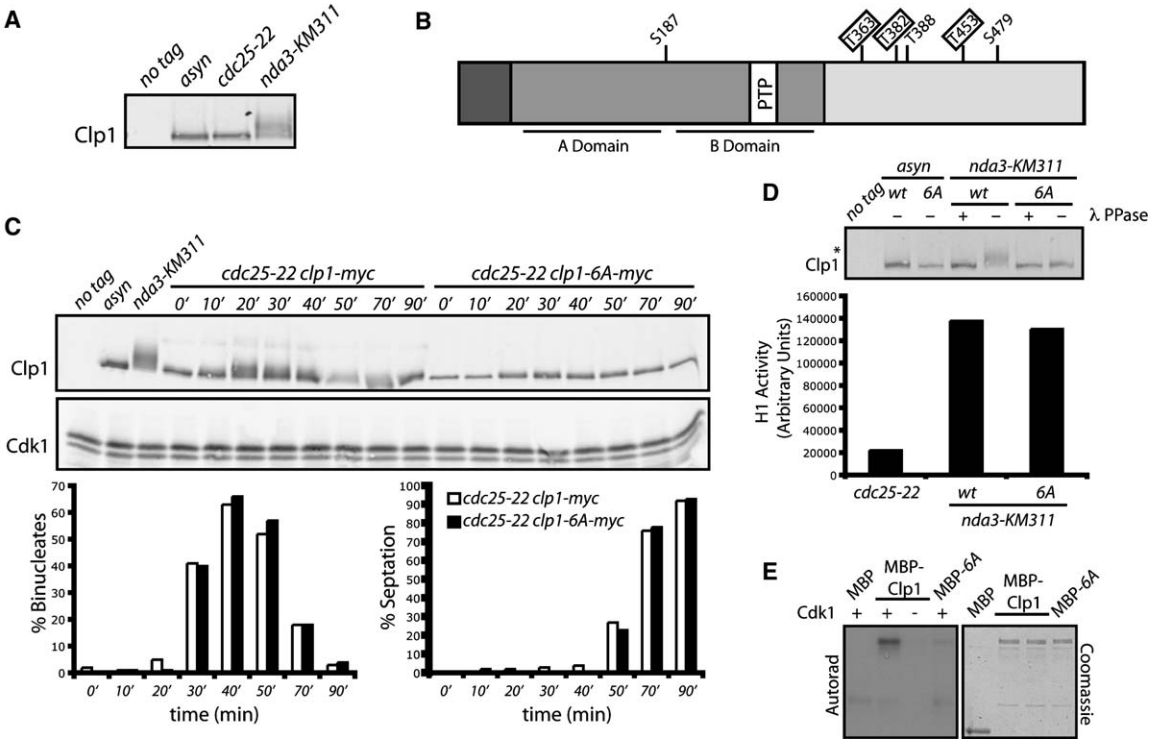

Figure 1. Cdk1 Phosphorylates Clp1 during Early Mitosis

(A) Clp1 levels and phosphorylation status were determined by immunoblot analysis in wild-type (*no tag*) and *clp1-myc* (*asyn*) cells grown at 32°C, *cdc25-22 clp1-myc* cells arrested at 36°C, and *nda3-KM311 clp1-myc* cells arrested at 18°C.

(B) Schematic representation of Clp1 depicting phosphorylation sites identified by mass spectrometric analysis of Clp1-TAP purified from *nda3-KM311 clp1-TAP* cells arrested at 18°C. Boxed sites represent those mutated in Clp1-*3A* and Clp1-*3D*.

(C) *cdc25-22 clp1-myc* and *cdc25-22 clp1-6A-myc* were synchronized at the G_2/M boundary at 36°C and released to the permissive temperature at 25°C. Samples were taken at the indicated time points, and Clp1 and Cdk1 levels (upper panel) and cell cycle stage (lower panel) were determined by immunoblot analysis and microscopy, respectively.

(D) Clp1 phosphorylation status in the presence or absence (+/−) of lambda phosphatase (λ) was determined in wild-type (*no tag*), *clp1-myc* (*wt*), and *clp1-6A-myc* (6a) cells grown asynchronously (*asyn*) at 32°C, and *nda3-KM311 clp1-myc* and *nda3-KM311 clp1-6A-myc* cells arrested at 18°C. (Lower panel) Histone H1 kinase activity was determined in *cdc25-22*, *nda3-KM311 clp1-myc*, and *nda3-KM311 clp1-6A-myc* cells arrested at the restrictive temperatures.

(E) Recombinant MBP, MBP-Clp1, and MBP-Clp1-*6A* were phosphorylated in vitro by kinase active (+) or kinase dead (−) Cdk1.

present in the noncatalytic carboxyl terminus (Figure 1B). These sites were mutated to nonphosphorylatable Ala residues (*6A*) and used to replace the endogenous *clp1*$^+$ locus. The resulting *clp1-6A* strain produced a Clp1 mutant severely impaired in mitotic phosphorylation (Figures 1C and 1D), although cell cycle progression, Cdk1 histone H1 kinase activity, and mitotic redistribution were largely unaffected (Figures 1C and 1D, and Figure S1; see the Supplemental Data available with this article online). Since this result suggested that Cdk1 phosphorylates Clp1 in vivo, we tested recombinant Cdk1 for its ability to phosphorylate recombinant Clp1 in vitro. While kinase active Cdk1 (*KA*), but not catalytically inactive Cdk1 (*KD*), phosphorylated recombinant Clp1 very well, Cdk1-*KA* was unable to phosphorylate MBP alone or the Clp1-*6A* mutant efficiently (Figure 1E).

Clp1 Controls Its Own Dephosphorylation

Cdk1 and Cdc14 phosphatases both target Ser and Thr residues (in the case of Cdc14, only when phosphorylated) with an additional Pro at the +1 position (Gray et al., 2003; Kaiser et al., 2002). Because Cdk1 sites were phosphorylated on Clp1, the intriguing possibility was raised that Clp1 controlled its own dephosphorylation. To examine the kinetics of Clp1 dephosphorylation, we utilized the *nda3-KM311* mitotic mutant to block cells with Clp1 in the hyperphosphorylated state. These cells were then released to the permissive temperature and allowed to cycle synchronously. As cells exited mitosis, Clp1 became dephosphorylated with kinetics similar to previous reports of Cdk1 inactivation (Figure 2A) (Wolfe and Gould, 2004; Esteban et al., 2004).

In order to test whether or not Clp1 phosphatase activity was required for the observed dephosphorylation, we generated phosphatase dead (*PD*) mutants (*C286S* and *D257A*) and replaced the wild-type *clp1*$^+$ locus with them. The two mutant strains were phenotypically identical, and the Clp1-*C286S* and Clp1-*D257A* proteins both lacked detectable phosphatase activity (data not shown and Figure S2). Therefore, they were used interchangeably throughout these studies. Clp1-*PD* remained in the hyperphosphorylated state when released from a prometaphase arrest even as cells exited mitosis and underwent septation (Figure 2B and Figure S3). In fact, Clp1-*PD* ran in the hyperphosphorylated state constitutively, comparable to mitotic Clp1, even during

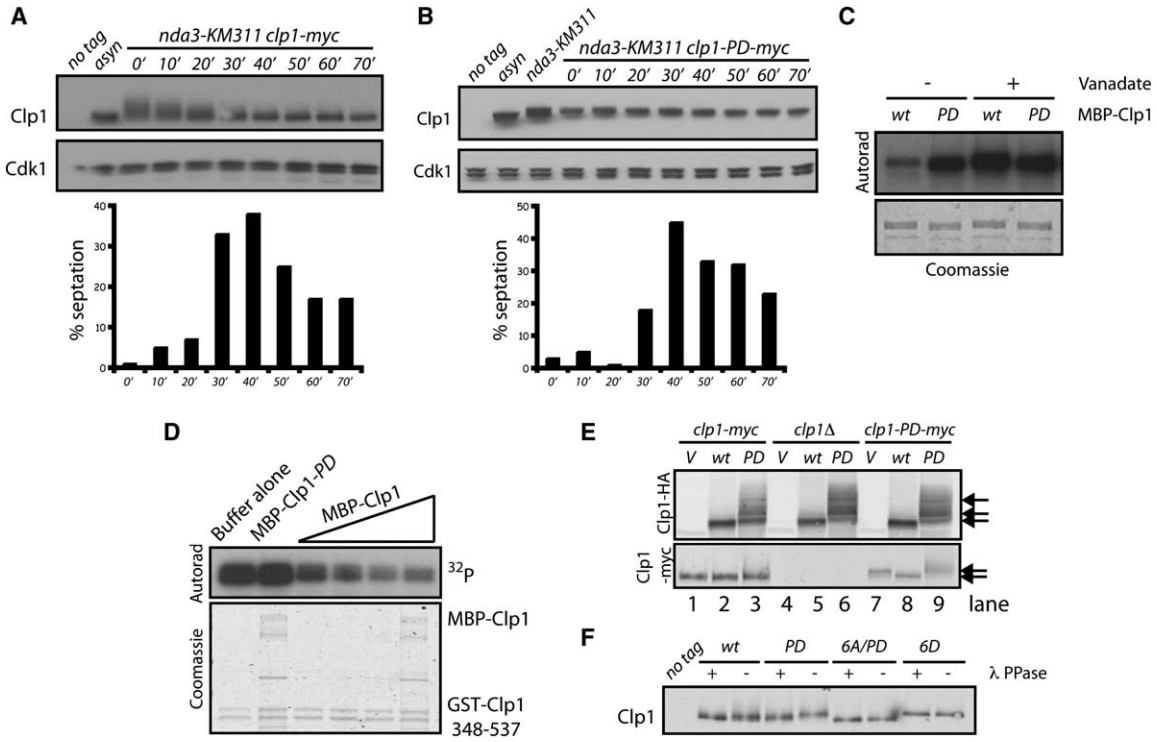

Figure 2. Clp1 Autodephosphorylates during the Exit from Mitosis

(A and B) (A) *nda3-KM311 clp1-myc* and (B) *nda3-KM311 clp1-D257A-myc* (*PD*) cells were arrested at 18°C, then released to 32°C to allow a synchronous progression out of mitosis. Samples were prepared at the indicated time points, and Clp1 and Cdk1 levels (upper panel) and septation index (lower panel) were determined by immunoblot analysis and light microscopy, respectively. Wild-type Clp1-myc from cells grown asynchronously (*asyn*) (A and B) and from arrested *nda3-KM311* cells (B) were also included as controls.

(C) MBP-Clp1 (*wt*) and MBP-Clp1-*PD* were phosphorylated in vitro with Cdk1 in the absence or presence (−/+) of 100 μM sodium vanadate.

(D) GST-Clp1 348–537 was phosphorylated in vitro by Cdk1, washed, and then incubated in the presence of buffer alone, 100 ng of MBP-Clp1-*PD*, or 0.8, 4, 20, or 100 ng of MBP-Clp1.

(E) *clp1-myc*, *clp1Δ*, and *clp1-D257A-myc* (*PD*) strains transformed with *pRHA* (*V*), *pRHA:clp1* (*wt*), and *pRHA:clp1-PD* (*PD*) vectors were induced to express HA-Clp1. Clp1-myc and HA-Clp1 phosphorylation states were determined via immunoblot analysis. Arrows indicate position of different phosphorylated species.

(F) Wild-type (*no tag*), *clp1-myc* (*wt*), *clp1-D257A-myc* (*PD*), *clp1-6A/D257A-myc* (*6A/PD*), and *clp1-6D-myc* (*6D*) strains were grown at 32°C, and Clp1 phosphorylation status after incubation in the presence or absence (+/−) of lambda phosphatase (λ) was determined via immunoblot analysis.

interphase (Figure 2F and data not shown). These data suggest that Clp1 autodephosphorylates during mitotic exit. Further, consistent with the idea that Clp1 autodephosphorylates Cdk1 sites of phosphorylation, mutation of the six Cdk1 phosphorylation sites in Clp1-*PD* abolished the gel shift (Figure 2F).

We next asked whether Clp1 could autodephosphorylate in vitro. Incubation of Clp1-*PD* with Cdk1 yielded increased radiolabel incorporation relative to wild-type Clp1 acting as the phospho-acceptor (Figure 2C). This could stem from enhanced phosphorylation of Clp1-*PD* or from Clp1 autophosphatase activity during the course of the reaction. To distinguish between these possibilities, we added sodium vanadate, an inhibitor of tyrosine family phosphatases, to the kinase reactions. Wild-type Clp1 retained the same amount of radiolabel in the presence of the inhibitor as Clp1-*PD* (Figure 2C). We conclude that Clp1 autodephosphorylates in vitro as well as in vivo.

To determine if Clp1 has the ability to autodephosphorylate in *trans*, we phosphorylated a C-terminal fragment of Clp1 lacking the phosphatase domain. This phosphorylated fragment was then used as an in vitro substrate for recombinant Clp1. Whereas increasing concentrations of Clp1 reduced the amount of radiolabel incorporation, the highest concentration of Clp1-*PD* failed to dephosphorylate this fragment (Figure 2D). Similar results were obtained when full-length, phosphorylated Clp1-*PD* was used as the substrate (Figure S4).

To test if *trans* autodephosphorylation could also occur in vivo, *HA-clp1*$^{+}$ or *HA-clp1-PD* was expressed under control of the inducible *nmt* promoter in *clp1Δ*, *clp1*$^{+}$, or *clp1-PD* strains in which endogenous *clp1* genes produced *myc*-tagged fusions, and the phosphorylation states of the different Clp1 proteins were determined through immunoblotting. HA-Clp1-*PD* was hyperphosphorylated in all strains (Figure 2E, upper panel). However, a significant increase in phosphorylation was seen when *HA-clp1-PD* was expressed in the absence of endogenous phosphatase activity (Figure 2E, upper panel; compare top two arrows in lanes 3, 6, and 9). Significantly, expression of *HA-clp1*$^{+}$, but not *HA-clp1-PD*, caused endogenous Clp1-*PD* to accumulate in the hypophosphorylated state (Figure 2E, lower panel, lane 8). These results indicate that *trans* autodephosphorylation can also occur in vivo.

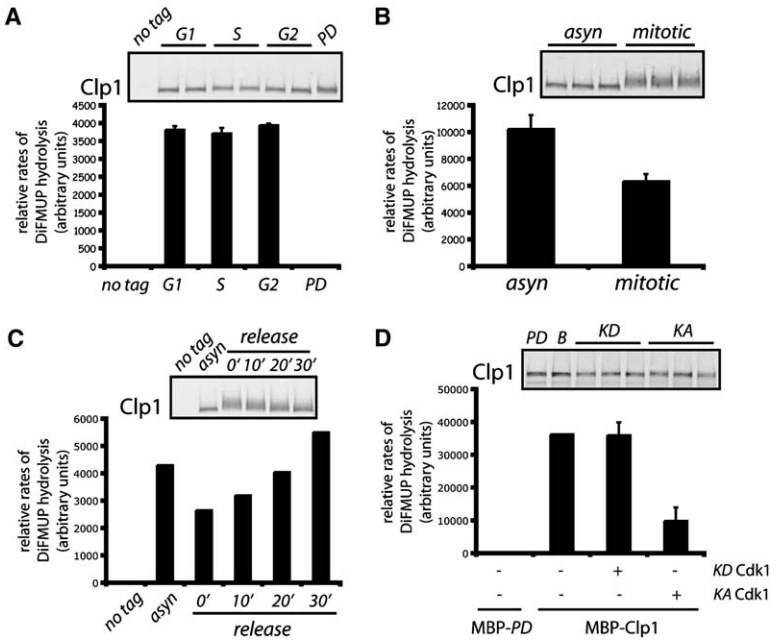

Figure 3. Cdk1 Phosphorylation of Clp1 Attenuates Its Activity

(A) Wild-type (*no tag*) and *clp1-C286S-myc* (*PD*) cells were grown at 32°C, *cdc10-v50 clp1-myc* (*G1*) and *cdc25-22 clp1-myc* (*G2*) cells were arrested at 36°C, and *clp1-myc* (*S*) cells were grown at 25°C in the presence of 12 mM hydroxyurea. Clp1 phosphatase activity was determined via its ability to hydrolyze DiFMUP, and its levels were determined by immunoblot analysis. Reactions were performed in duplicate for standard error analysis. Data are representative of three such independent experiments.

(B) *clp1-myc* (*asyn*) cells were grown at 32°C, and *nda3-KM311 clp1-myc* (*mitotic*) cells were arrested at 18°C. Clp1 phosphatase activity was determined as in (A), and its levels and phosphorylation status were determined by immunoblot analysis. Reactions were performed in triplicate to generate standard error. Data are representative of three such independent experiments.

(C) *nda3-KM311 clp1-myc* cells were arrested at prometaphase by incubation at 18°C. Cells were then released to 32°C, and samples were taken at the indicated time points. Clp1 levels and phosphorylation status were determined by immunoblot analysis, and activity was determined as in (A). Data are representative of at least three independent experiments.

(D) MBP-Clp1 or MBP-Clp1-*PD* was incubated in the presence of buffer (*B*), kinase active (*KA*), or kinase dead (*KD*) Cdk1 and ATP-γ-S, washed, and then assayed for activity as in (A). MBP-Clp1 levels were determined by immunoblot analysis. Reactions in the presence of Cdk1 were performed in triplicate to generate standard error. Data are representative of two independent experiments.

Mitotic Phosphorylation of Clp1 Reduces Its Catalytic Activity

Despite Clp1's release from the nucleolus upon entry into mitosis (Trautmann et al., 2001; Cueille et al., 2001), both Clp1 and its substrate Cdc25 are in the hyperphosphorylated state prior to anaphase (Figure 1A) (Wolfe and Gould, 2004; Esteban et al., 2004). This suggested that Clp1 activity is restrained prior to sister chromatid separation. To evaluate the contribution of Clp1 mitotic phosphorylation on its catalytic activity, we assayed the effects of phosphorylation in vitro. First, immunopurified Clp1 from *S. pombe* cells was examined for its ability to hydrolyze the artificial substrate, DiFMUP. Clp1 presence in the immune complex as well as intact active site Cys286 and Asp257 were required to hydrolyze DiFMUP (Figure 3A and data not shown). Continuous assays revealed that Clp1 activity from different interphase blocks was detectable and varied little (Figure 3A). By analogy with *S. cerevisiae* Cdc14 (Shou et al., 1999; Visintin et al., 1999), we would have expected that a nucleolar binding partner analogous to Net1/Cfi1 would have inhibited Clp1 during nucleolar localization, as occurs during interphase. Should there exist a similar competitive inhibitor, the interaction must be lost under our lysis conditions. In contrast, the rate at which mitotic Clp1 hydrolyzed DiFMUP was reduced by 40% of that observed in asynchronous samples in which upwards of 90% of the cells are in interphase (Figure 3B). Because Clp1 is hyperphosphorylated at this point (Figures 1A, 2A, and 3B), these data suggest that Clp1 mitotic phosphorylation might inhibit its activity.

Should this be the case, we would expect that autodephosphorylation during mitotic exit would restore Clp1 activity to interphase levels. To examine this possibility, we performed an *nda3-KM311* block-and-release experiment. As cells exited mitosis and underwent septation (data not shown), Clp1 became dephosphorylated and its catalytic activity increased (Figure 3C). To confirm that Cdk1 phosphorylation inhibits Clp1 phosphatase activity, we turned to an in vitro assay containing only purified recombinant proteins. To circumvent the issue that recombinant Clp1 autocatalytically removes Cdk1 phosphorylation events, we included in the kinase reactions the nonhydrolyzable ATP analog, ATP-γ-S. After incubation with Cdk1, Clp1's ability to hydrolyze DiFMUP was reduced to one-third that achieved upon incubation in the presence of either buffer alone or catalytically inactive Cdk1 (Figure 3D). Similarly, when an excess of kinase active, but not kinase dead, Cdk1 was included alongside hydrolyzable ATP in the phosphatase assay, Clp1 activity was compromised to the same extent (Figure S5). Combined with our analysis of Clp1 activity purified from *S. pombe* cells, these data confirm that Clp1 mitotic phosphorylation by Cdk1 reduces its catalytic activity. The extent to which phosphorylated Clp1 is inhibited in vivo may be even more substantial, as heterogeneous phosphorylated forms exist in both of our analyses.

Having established that Cdk1 phosphorylation attenuates Clp1 catalytic activity, we next addressed what effect this has on Clp1 function in vivo. Complicating this examination, we found that recombinant Clp1-*6A* had significantly less phosphatase activity in vitro compared with wild-type Clp1, suggesting that at least one of the mutations disrupted a function unrelated to phosphorylation (Figure S2). Therefore, we generated subsets of mutations at the six phosphorylation sites in an effort to identify a mutant that eliminated Clp1 mitotic

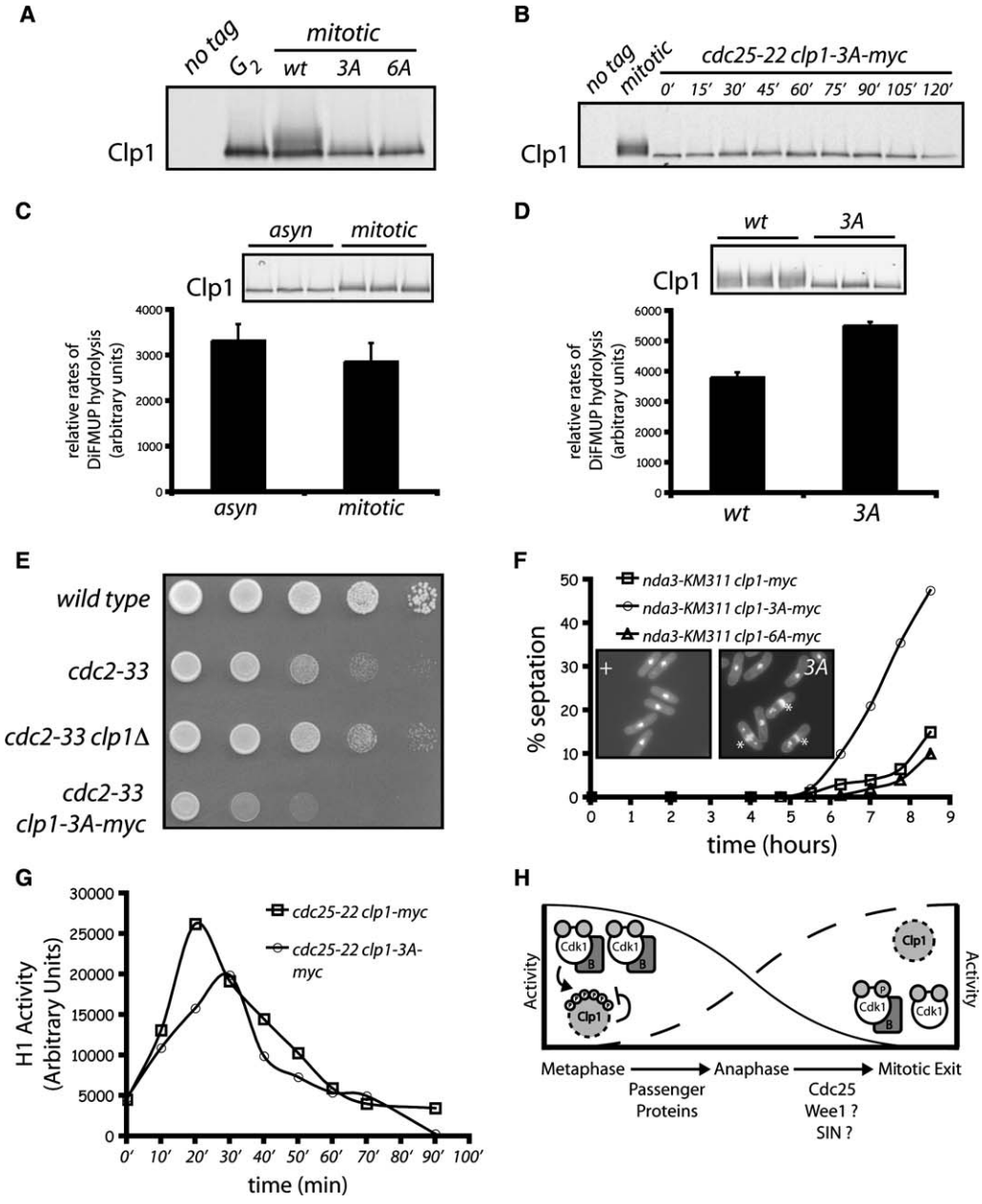

Figure 4. Preventing Clp1 Mitotic Phosphorylation Eliminates Mitotic Inhibition

(A) Wild-type (*no tag*) cells were grown at 32°C, *cdc25-22 clp1-myc* (*G2*) cells were arrested at 36°C, and *nda3-KM311 clp1-myc* (*wt*), *nda3-KM311 clp1-3A-myc* (*3A*), and *nda3-KM311 clp1-6A-myc* (*6A*) cells were arrested in mitosis at 18°C. Clp1 levels and phosphorylation status were determined by immunoblot analysis.

(B) Wild-type (*no tag*) cells were grown at 32°C, *nda3-KM311 clp1-myc* (*mitotic*) cells were grown at 18°C, and *cdc25-22 clp1-3A-myc* cells were arrested at the G_2/M boundary prior to being released at 25°C for the indicated times. Clp1 levels and phosphorylation status were determined through immunoblot analysis.

(C) *clp1-3A-myc* (*asyn*) cells were grown at 32°C, and *nda3-KM311 clp1-3A-myc* (*mitotic*) cells were arrested in prometaphase at 18°C. Clp1 phosphatase activity was determined, and its levels and phosphorylation status were determined by immunoblot analysis. Reactions were performed in triplicate to generate standard error. Data are representative of three independent experiments.

(D) *nda3-KM311 clp1-myc* (*wt*) and *nda3-KM311 clp1-3A-myc* (*3A*) cells were arrested in prometaphase at 18°C. Clp1 phosphatase activity was determined, and its levels and phosphorylation status were determined by immunoblot analysis. Reactions were performed in triplicate to generate standard error. Data are representative of three independent experiments.

(E) Wild-type, *cdc2-33*, *cdc2-33 clp1Δ*, and *cdc2-33 clp1-3A-myc* were serially diluted (1/10) onto YE medium and incubated at 33°C for 48 hr.

(F) *nda3-KM311 clp1-myc*, *nda3-KM311 clp1-3A-myc*, and *nda3-KM311 clp1-6A-myc* cells were grown overnight at 32°C and synchronized by centrifugal elutriation prior to growth arrest in prometaphase at 18°C. Septation index was determined by light microscopic analysis of 400 cells, and ethanol-fixed cells were stained with methyl blue and DAPI to visualize septa and nuclei, respectively. Asterisks indicate "cut" cells.

(G) *cdc25-22 clp1-myc* and *cdc25-22 clp1-3A-myc* were synchronized at the G_2/M boundary at 36°C and released to the permissive temperature at 25°C. Samples were taken at the indicated time points, and H1 activity of Cdk1 immunoprecipitates was determined and quantified.

(H) During metaphase, Cdk1 antagonizes Clp1 activity through phosphorylation of its C terminus. A drop in Cdk1 activity at anaphase onset allows Clp1 to autodephosphorylate, become fully active, disrupt the Cdk1 autoamplification loop at mitotic exit, and potentiate SIN signaling.

hyperphosphorylation while retaining wild-type phosphatase activity in vitro. Only one of the mutants, designated Clp1-*3A* (Figure 1B), was significantly impaired for mitotic phosphorylation (Figures 4A, 4B, and data not shown), and in recombinant form it possessed wild-type activity in vitro (Figure S2). Clp1-*3A* activity was resistant to mitotic inhibition, being reduced by only 15% when compared to its asynchronous control (Figure 4C). Also, its activity in mitosis was elevated 30% above the wild-type control (Figure 4D). These data suggested that it might be a gain-of-function mutant in vivo. Several lines of evidence support this prediction: (1) whereas the temperature sensitive *cdc2-33* allele is partially rescued by *clp1*$^{+}$ deletion (Esteban et al., 2004), the *cdc2-33 clp1-3A* double mutant is inviable at the same reduced restrictive temperature (Figure 4E); (2) *clp1-3A nda3-KM311* cells arrested in prometaphase fail to prevent septation (Figure 4F); and (3) Cdk1 activation is delayed, less robust, and attenuated more rapidly in Clp1-*3A* cells when compared to wild-type (Figure 4G and Figure S6). Cumulatively, these data indicate that Cdk1-mediated inhibition of Clp1 catalytic activity during early mitosis ensures the proper kinetics of mitotic progression and safeguards the integrity of the genome by delaying the onset of cytokinesis prior to chromosome segregation.

Discussion

Although Clp1 is released from the nucleolus in early mitosis and is potentially free to dephosphorylate substrates (Trautmann et al., 2001; Cueille et al., 2001), mitotic substrates remain hyperphosphorylated until mitotic exit (Wolfe and Gould, 2004; Esteban et al., 2004). Our data suggest that Clp1 phosphatase activity is attenuated during this time through inhibitory phosphorylation by Cdk1. We have identified mitotic-specific phosphorylation events occurring at Cdk1 consensus sites, and Cdk1 has the ability to phosphorylate these sites in vitro. Additionally, we have uncovered a positive feedback loop regulating Clp1 activation at the exit from mitosis: Clp1 autocatalytically removes the inhibitory phosphorylations to restore its full catalytic potential and prevents further inhibition by helping to attenuate Cdk1 activity. Mitotic phosphorylation likely acts to restrain Clp1 targeting of key substrates until a drop in Cdk1 activity has occurred at anaphase onset. This is another example of Cdk1 inhibition of its negative regulators when it is fully active (Visintin et al., 1998; Jaspersen et al., 1999; Verma et al., 1997).

Clp1 Mitotic Phosphorylation—Analogous to FEAR?

We propose that full Clp1 activation occurs in at least two steps. The first occurs when cells enter mitosis and Clp1 is released from the nucleolus to concentrate at the contractile ring and kinetochores (Trautmann et al., 2001, 2004; Cueille et al., 2001). At this stage, some degree of Clp1 catalytic activity must exist since Clp1 is required at kinetochores for monitoring chromosome biorientation (Trautmann et al., 2004). The second step occurs during anaphase and involves autocatalytic reversal of Cdk1-mediated inhibitory phosphorylation events, resulting in increased Clp1 activity. During this phase, Clp1 is able to target Cdc25 for dephosphorylation, disrupt the Cdk1 autoamplification loop, and potentiate SIN signaling (Wolfe and Gould, 2004; Esteban et al., 2004). Modification by other kinases and phosphatases might also influence Clp1 activity.

The regulation of Clp1 in *S. pombe* resembles the stepwise activation of *S. cerevisiae* Cdc14. The FEAR and MEN networks collaborate to promote the full release of Cdc14 from its nucleolar inhibitor (Stegmeier and Amon, 2004). In addition to regulating Cdc14 nucleolar localization, these pathways target Cdc14 to distinct substrates required for progression through either early or late anaphase. For example, only MEN-activated Cdc14 is able to fully combat Cdk1 activity by targeting the Cdk1 inhibitor Sic1 for stabilization and the APC activator Cdh1/Hct1 for activation, thus restricting mitotic exit to late anaphase (Visintin et al., 1998). Such temporal regulation could be accounted for by varying substrate accessibility and/or substrate affinity. Strikingly, whereas FEAR-released Cdc14 is able to interact with the MEN GAP component, Bfa1, it fails to dephosphorylate it until MEN has become active (Pereira et al., 2002). Similarly, we have found that while Clp1 interacts with Cdc25 during early mitosis (B.A.W. and K.L.G., unpublished data), efficient Cdc25 dephosphorylation only occurs during anaphase (Wolfe and Gould, 2004; Esteban et al., 2004). These data suggest that there remains an additional layer of regulation influencing the timing of Cdc14 family substrate dephosphorylation.

Our data suggest that in *S. pombe*, Clp1 mitotic phosphorylation by Cdk1 may play such a regulatory role and contribute to the timing of substrate dephosphorylation (Figure 4H). Because Clp1 activity is attenuated in response to mitotic phosphorylation and only becomes fully active after autodephosphorylation at mitotic exit, a gradient of Clp1 activity is formed which could specify the order of substrate dephosphorylation by allowing only those substrates with high affinities to be dephosphorylated early in mitosis. Under situations that necessitate repeated rounds of phosphorylation and dephosphorylation such as might occur to achieve chromosome biorientation (Trautmann et al., 2004), this may be a critical mode of restraining the finality of certain events. As *h*Cdc14A and *X*Cdc14 are similarly hyperphosphorylated during early mitosis (Kaiser et al., 2002, 2004), this may represent a conserved mechanism whereby Cdc14 protein substrate selectivity is temporally determined.

Experimental Procedures

Strains, Media, and Methods

For *cdc25-22* block-and-release experiments, cells were shifted to the restrictive temperature at 36°C for 4 hr. Cells were then released to the permissive temperature at 25°C to undergo a synchronous mitosis. For *nda3-KM311* block-and-release experiments, cells were shifted to the restrictive temperature at 18°C for 7 hr, and then released to the permissive temperature at 32°C to undergo a synchronous anaphase.

Cytology and Microscopy

To visualize DNA and septa, cells were fixed with ethanol and stained with DAPI (4,6-diamidino-2-phenylindole) as described (Chang et al., 2001) and methyl blue. At least 200 cells were scored for number of nuclei. For live cell imaging, cells were grown at 18°C and visualized using an Ultraview LCI confocal microscope equipped with a 488 nm Ar ion laser (Perkin-Elmer). Z series optical

sections were taken at 0.5 μm spacing, and images were captured using Ultraview LCI software (version 5.2; Perkin-Elmer) and processed using Volocity software (version 1.4.2; Improvision).

Protein Methods

Phosphatase assays using lambda phosphatase were performed as described (Wolfe and Gould, 2004). Immunoblot analysis was performed as described (Tomlin et al., 2002), except primary antibodies were detected with secondary antibodies coupled to either horseradish peroxidase for enhanced chemiluminescence or to Alexa Fluor 680 (Invitrogen) for scanning on an Odyssey machine (LI-COR). Quantifications of protein intensities were performed using Odyssey (LI-COR) version 1.2.

In Vitro Kinase and Phosphatase Assays

All recombinant bacterially produced proteins were purified on either glutathione beads (GST) or amylose beads (MBP) as described (Tomlin et al., 2002). Approximately 50 ng of recombinant Cdk1 kinase complex, purified from baculovirus infected *Sf9* cells as described (Yoon et al., 2002), was used to phosphorylate approximately 0.5–1 μg of recombinant MBP-Clp1 and mutants in HB15 buffer supplemented with 50 μM cold ATP and 5 μCi [γ-^{32}P]ATP. Reactions were incubated at 30°C for 20 min and terminated by the addition of sample buffer. Samples were boiled and separated on SDS-PAGE. Coomassie staining or autoradiography was used for detection of proteins. Histone H1 kinase assays were performed on immunoprecipitated Cdk1 as described (Gould et al., 1991).

For phosphatase assays, recombinant GST-Clp1 containing amino acids 348–537 was phosphorylated by kinase active Cdk1 as before on glutathione beads. Beads were washed extensively in TB1 buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton-X100) and eluted in TB1 buffer containing 20 mM glutathione. Eluted phosphorylated protein was incubated in the presence of recombinant phosphatase at 30°C for 30–45 min in phosphatase assay buffer (50 mM Imidazole [pH 6.9], 1 mM EDTA, 1 mM DTT). Reactions were terminated by the addition of sample buffer and boiling, followed by separation on SDS-PAGE and visualization by Coomassie staining and autoradiography.

DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate) (Invitrogen) continuous assays were performed on 25–50 ng of recombinant MBP-Clp1 and mutants purified as described (Tomlin et al., 2002) or on Clp1-myc immune complexes obtained from 20 OD_{595} of cells as described (Gould et al., 1991), with the exception that cells were lysed in a modified NP-40 buffer lacking sodium vanadate but containing instead 60 mM β-glycerophosphate. Clp1-myc immune complexes were resuspended in phosphatase assay buffer supplemented with bovine serum albumin (New England Biolabs) to 250 μg/ml, in a 96-well plate. DiFMUP was added via a FlexStation (Molecular Devices) to a final concentration of 250 μM, and fluorescence was monitored at 30°C for 10 to 20 min every 30 s with excitation at 385 nm and emission measured at 455 nm. Fluorescent readings were plotted using Excel, and the rates of the reaction were determined using linear regression analysis. Protein quantifications for normalization were measured from 1/5 of the assay input using Odyssey software (LI-COR). For the kinase/phosphatase reactions containing ATP-γ-S, bead bound *r*MBP-Clp1 was phosphorylated by recombinant Cdk1 in the presence of 1 mM ATP-γ-S sequentially four times at 30°C for 120 min. Prior to the phosphatase reactions, *r*MBP-Clp1-containing beads were washed extensively to remove recombinant Cdk1 and ATP-γ-S. For the phosphatase reactions containing simultaneous inclusion of Cdk1 and Clp1, approximately 10-fold excess kinase dead or kinase active Cdk1 was incubated with recombinant Clp1 in phosphatase assay buffer containing 0.5 mM ATP and 10 mM $MgCl_2$ for 10 min at 30°C prior to the addition of DiFMUP.

Supplemental Data

Supplemental Data include six figures, one table and Supplemental Experimental Procedures and are available at http://www.developmentalcell.com/cgi/content/full/11/3/423/DC1/.

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